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THE RELATION BETWEEN SERUM RESISTANCE AND VIRULENCE.*

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A NUMBER of investigations into the interactions between bacteria and serum in vitro have been reported within the last few years. In these investigations bacteria have been subjected to the action of their own immune serum, of other immune sera, of active normal serum, and of inactive normal serum. Most, if not all, of the experiments previously reported have been upon certain pathogenic forms, especially B. typhosus, B. anthracis, and the cholera vibrio.

Increase in serum resistance together with increased virulence has been observed in B. typhosus by Walker[1] and Steinhardt.[2] Walker secured a markedly increased serum resistance and an increase in virulence for both rabbits and guinea-pigs by passage through normal rabbit blood. Steinhardt observed increased serum resistance in only one of four series passed through normal horse serum. In this one series of 11 passages the virulence was increased sevenfold. In another series of 43 passages, virulence was increased twofold, but there was no increase in serum resistance. Serum resistance did not develop in the other two series, no virulence tests being made. Shaw[3] found a fourfold increase of virulence for B. anthracis after five passages through normal human serum. While he made no experiments to determine the serum resistance, he concluded from the character of the growths observed during several passages, that the organism underwent a gradual immunization against the action of the serum.

Increased serum resistance of B. anthracis to normal rabbit serum without increase in virulence has been reported by Sacharoff.[4] Cohn[5] obtained increased resistance in B. typhosus by

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[1] *Brit. Med. Jour.*, 1902, 2, p. 1199.

[2] *Jour. Med. Res.*, 1905, 13, p. 409.

[3] *Brit. Med. Jour.*, 1903, 1, p. 1074.

[4] *Centralb. f. Bakt.*, 1904, 37, p. 411.

[5] *Ztschr. f. Hyg.*, 1903, 45, p. 61.

passage through active rabbit serum, but no resistance when passed through inactivated serum. The same results were obtained by Trommsdorf[1] with B. typhosus and with the cholera vibrio. Neither of the latter investigators tested for virulence.

Increased virulence of cholera vibrios after passage through normal horse serum and normal guinea-pig serum has been reported by Hamburger.[2]

On the whole, examination of the literature upon experiments with normal bactericidal serum shows that the results have been rather irregular. Increase in serum resistance is noted in most instances, but is not always accompanied by increase in virulence.

In the experiments here reported, three relatively nonpathogenic organisms were used; namely, B. prodigiosus, B. proteus vulgaris, and B. fluorescens nonliquefaciens. All were taken from stock cultures that had been on artificial media in this laboratory for several years, and were found to exhibit typical cultural characteristics.

The method of serum culture was similar to that of Ainley Walker.[3] The cultures were made upon slant agar, so slanted as to give approximately the same area to each surface. After 24 hours an emulsion of the growth was made in five c.c. of sterile normal salt solution. Dilutions ranging from one in one thousand to one in ten million were made of this emulsion, and two c.c. of each dilution placed in a small serum tube. Two c.c. of fresh normal rabbit serum were added to each tube, the mixture incubated for three hours, and then plated. At the end of 24 hours a transfer from plate to slant agar was made from the highest dilution which showed growth, thus securing those organisms which had been subjected to the strongest action of the serum. After several passages, to obtain a more concentrated action of the serum, three c.c. of serum were added to each two c.c. of suspension, and later four c.c. A control or parallel series for each organism was run on slant agar.

First series.—This series consisted of 10 passages by the method described above. After three daily transfers on agar, following the last passage through serum, as nearly uniform emulsions as possible were made of each serum-grown culture and of its control. To three c.c. of each emulsion were added three c.c. of normal rabbit serum, the two thoroughly mixed, $\frac{1}{10}$ c.c. immediately removed and plated to determine the initial bacterial content, and the remainder then placed in the thermostat. Subsequent platings at the intervals indicated in the table (Table 1) were made in the same manner.

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[1] *Archiv f. Hyg.*, 1900–01, 39, p. 31.

[2] *Wien. klin. Wchnschr.*, 1903, 16, p. 97.

[3] *Brit. Med. Jour.*, 1902, 2, p. 1199.

TABLE 1.

SERUM RESISTANCE AFTER 10 GENERATIONS IN ACTIVE SERUM.

HOURS	B. PRODIGIOSUS		B. PROTEUS VULGARIS		B. FLUORESCENS	
	Serum Culture	Agar Culture	Serum Culture	Agar Culture	Serum Culture	Agar Culture
0	43,600	30,200	53,800	61,200	33,500	40,000
1	24.200	22,700	45,400	40,500	30,100	32,500
3	36.300	28,000	80,000	54.300	9,500	28,000
6	50,000	47,000	120 000	110,000	10,200	21,000
12	110,000	97,000	225,000	262,500	46,000	35,200

The test for serum resistance shows that no one of the cultures has undergone any change as the result of the action of the serum. The serum-grown cultures possibly overcame the action of the serum slightly more rapidly, but the difference is so small that little importance can be attached to it.

One fact which attracts attention is the strong natural resistance of these organisms to the bactericidal action of the serum. According to Buxton,[1] one c.c. of normal rabbit serum may be expected to kill 1,000,000 typhoid bacilli, 50,000,000 paratyphoid bacilli, and 100,000,000 cholera vibrios. By experiment I determined that one c.c. of normal rabbit serum would kill only 300,000 proteus vulgaris bacilli, and 350,000 prodigiosus bacilli. This serum was tested on B. typhosus and found to have the usual bactericidal action on this organism. More evidence of the low bactericidal action upon these nonpathogenic organisms was shown by the fact that even during the first passages growth invariably occurred in the highest dilutions where comparatively few bacilli were subjected to the action of the serum.

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[1] *Jour. Med. Res.*, 1905, 13, p. 305.

The first animal inoculation of this series was made 10 days after the last passage through serum. Four c.c. of a 24 hour broth culture of B. prodigiosus was injected into the peritoneal cavity of a full-grown rabbit. At the end of 10 hours the animal was found dead. Cultures were made from the peritoneal cavity and heart, and the organism isolated in pure culture. Further animal inoculations with this series were now made (Table 2). All inoculations were of 24 hour broth cultures and were made intraperitoneally.

TABLE 2.

ANIMAL INOCULATIONS AFTER 10 GENERATIONS IN NORMAL SERUM.

CULTURE		AMT. OF 24 HOUR BROTH CULTURE	ANIMAL AND WEIGHT		RESULT
			Rabbits	Guinea-Pigs	
B. prodigiosus	Control	8 c c.	1,730 gms.	...	Slightly sick; recovered
	Control	6	1,370	...	No effect
	Serum culture ..	4	1,345	...	Dead in 10 hours
	Serum culture ..	2	1,550	...	Very sick; recovered
	Control	4	.....	365 gms.	No effect
	Serum culture ..	4	.....	354	" "
B. proteus vulgaris	Control	6 c.c.	1,545 gms.	...	No effect
	Serum culture ..	4	1,640	...	Dead in 20 hours
	Serum culture ..	2	1,720	...	" " 14 days
	Control	4	.....	360 gms.	No effect
	Serum culture ..	4	.....	370	" "
B. fluorescens	Control	8 c.c.	1,640 gms.	...	No effect
	Serum culture ..	4	1,620	...	Dead in 12 hours
	Serum culture ..	2	1,700	...	Sick; recovered
	Control	4	.....	415 gms.	No effect
	Serum culture ..	4	.....	422	" "

There was a very marked increase in the virulence of each organism for rabbits, but no increase for guinea-pigs was detected.

Second active serum series.—In order to test further the relation between serum resistance and virulence, the series through active serum was repeated. This second series consisted of a total of 20 passages and extended over a period of about 12 weeks.

After the first three passages through the serum, the cultures were tested to determine if, at this stage, there was any evidence of serum resistance. It was found that each serum-grown culture was slightly less resistant to the action of the serum than was its control. Evidence of this fact was also seen in the slant cultures taken from the plates following each passage, after a larger number of passages the slant cultures being noticeably more vigorous than at first. The power of pigment production by B. prodigiosus was greatly enhanced. The serum culture would produce an amount of pigment in 18 hours which it would take the control culture 48 hours to equal.

After the last of the 20 passages the cultures were transferred daily on agar for three days before making the resistance and virulence tests.

Table 3 gives the serum resistance after 20 generations in active serum.

TABLE 3.

SERUM RESISTANCE AFTER 20 GENERATIONS IN ACTIVE SERUM.

HOURS	B. PRODIGIOSUS		B. PROTEUS VULGARIS		B. FLUORESCENS	
	Serum Culture	Agar Culture	Serum Culture	Agar Culture	Serum Culture	Agar Culture
0	5,200	3,000	9,000	4,300	3,300	10,000
1	600	600	4,500	2,800	3.000	8,000
3	950	400	8,000	8,600	900	7,500
6	1,500	1,600	30,000	40,000	1,000	5,000
12	30,000	28,000	60,000	65,000	10,000	8,000

In this series after 20 generations, as in the first series after 10 generations, the serum-grown cultures have developed no appreciable resistance to the bactericidal action of the serum. The results from this standpoint are entirely negative.

As before, both rabbits and guinea-pigs were inoculated (Table 4). All inoculations were made intraperitoneally with 24 hour broth cultures.

For rabbits the M. L. D. of B. prodigiosus had been decreased from near eight c.c. to about one c.c.; of B. proteus vulgaris, from over six c.c. to two c.c.; and for B. fluorescens there was probably a slight increase in virulence, although this was not definitely determined.

For guinea-pigs, the M. L. D. of B. proteus vulgaris alone had decreased, three c.c. being fatal, whereas four c.c. of the control was not fatal. More accurate determination was not made.

TABLE 4.

ANIMAL INOCULATIONS AFTER 20 GENERATIONS IN ACTIVE SERUM.

CULTURE		AMT. OF 24 HOUR BROTH CULTURE	ANIMAL AND WEIGHT		RESULT
			Rabbits	Guinea-Pigs	
B. prodigiosus	Control	8 c.c.	1,730 gms.	...	Slightly sick; recovered
	Control	6	2,405	...	No effect
	Serum culture ..	2	1,250	...	Dead in 4 hours
	Serum culture ..	2	1,185	...	" " 3 "
	Serum culture ..	1½	1,175	...	" " 48 "
	Serum culture ..	1	1,540	...	" " 14 days*
	Control	4	.....	365 gms.	No effect
	Serum culture ..	4	.....	335	" "
	Serum culture ..	4	.....	392	" "
B. proteus vulgaris	Control	6 c.c.	1,920 gms.	...	No effect
	Serum culture ..	4	2,110	...	Dead in 12 hours
	Serum culture ..	4	1,650	...	" " 12 "
	Serum culture ..	2	1,360	...	" " 16 days*
	Control	4	.....	360 gms.	No effect
	Serum culture ..	4	.....	285	Dead in 12 hours
	Serum culture ..	4	.....	340	" " 12 "
	Serum culture ..	3	.....	310	" " 12 "
B. fluorescens	Control	8 c.c.	1,640 gms.	...	No effect
	Serum culture ..	4	1,700	...	Slightly sick; recovered
	Control	4	.....	415 gms.	No effect
	Serum culture ..	4	.....	300	" "

*Organism isolated in all cases except those marked with asterisk.

Inactive serum series.—For comparison with the above results, a series of passages (20 generations) were made through rabbit serum inactivated by heating at 55–56° C. for one-half hour.

B. fluorescens was not tested for serum resistance, but the other two organisms show no change as a result of the passages (Table 5).

TABLE 5.

SERUM RESISTANCE AFTER 20 GENERATIONS IN INACTIVE SERUM.

HOURS	B. PRODIGIOSUS		B. PROTEUS VULGARIS	
	Serum Culture	Agar Culture	Serum Culture	Agar Culture
0	4,800	5,300	5,940	5,090
1	800	1,500	1,320	600
3	1,025	1,200	2,640	2,100
6	7,400	3,700	11,000	6,400
12	43,000	36,000	74,000	5,400

The animal inoculations with this series gave the following results.

TABLE 6.

Animal Inoculations after 20 Generations in Inactive Serum.

Culture		Amt. of 24 Hour Broth Culture	Animal and Weight		Results
			Rabbits	Guinea-pigs	
B. prodigiosus	Control..........	8 c.c.	1,730 gms.	...	Slightly sick; recovered
	Control..........	6	2,405	...	No effect
	Serum culture..	4	1,490	...	" "
	Serum culture..	4	.....	365 gms.	" "
B. proteus vulgaris	Control..........	6 c.c.	1,920 gms.	...	No effect
	Serum culture..	4	1,425	...	Very sick; recovered
	Serum culture..	4	1,400	...	Dead in 12 hours
	Serum culture..	4	1,375	...	" " 4 "
	Serum culture..	2	1,060	...	" " 4 "
	Control..........	4	.....	360 gms.	No effect
	Serum culture..	4	.....	335	Dead in 12 hours
	Serum culture..	4	.....	315	" " 12 "
B. fluorescens	Control..........	8 c.c.	1,640 gms.	...	No effect
	Serum culture..	4	1,430	...	Dead in 12 hours
	Serum culture..	2½	1,440	...	" " 14 days
	Control..........	4	.....	415 gms.	No effect
	Serum culture..	4	.....	305	Dead in 12 hours

There is here no indication of an increase in virulence of the inactive serum culture of B. prodigiosus for either rabbits or guinea-pigs. The virulence of B. proteus vulgaris, however, is very much increased for rabbits and is somewhat increased for guinea-pigs, the exact degree for the latter not being determined. B. fluorescens also shows an increased virulence for both rabbits and guinea-pigs.

SUMMARY OF RESULTS.

1. None of the three slightly pathogenic or "nonpathogenic" organisms (B. prodigiosus, B. proteus vulgaris, and B. fluorescens nonliquefaciens) showed any change in serum resistance as a result of passage through either active or inactive normal rabbit serum.

2. Upon passage through active rabbit serum all three organisms increased in virulence for rabbits, B. prodigiosus at least sixfold. B. proteus vulgaris and B. fluorescens did not give quite so high increases in virulence. B. proteus vulgaris and B. fluorescens increased in virulence for guinea-pigs, but B. prodigiosus did not.

3. Upon passage through inactive serum, B. prodigiosus showed no increase in virulence for either rabbits or guinea-

pigs. B. proteus vulgaris showed an increase for rabbits equal to that obtained when passed through active serum. B. fluorescens showed a greater increase in virulence for guinea-pigs than upon passage through active serum.

In conclusion I wish to express my gratitude to Dr. E. O. Jordan for his interest and valuable suggestions during the course of the work.